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Assessing the impact of *in-utero* exposures: potential effects of paracetamol on male reproductive development

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ABSTRACT

Human male reproductive disorders (cryptorchidism, hypospadias, testicular cancer and low sperm counts) are common and some may be increasing in incidence worldwide. These associated disorders can arise from subnormal testosterone production during fetal life. This has resulted in a focus on *in-utero* environmental influences that may result in reproductive effects on the offspring in later life. Over recent years, there has been a dramatic increase in the scientific literature describing associations between *in-utero* environmental exposures (e.g. industrial chemicals and pharmaceuticals) and subsequent reproductive outcomes in male offspring. This includes studies investigating a potential role for *in-utero* analgesic exposure(s) on the fetal testis; however, providing definitive evidence of such effects presents numerous challenges. In this review, we describe an approach to assessing the potential clinical relevance of *in-utero* (and postnatal) environmental exposures on subsequent male reproductive function using exposure to the analgesic paracetamol as an example.

Male Reproductive Disorders and Testicular Dysgenesis Syndrome

From the mid 1900's to the present date, many studies have reported evidence for an increasing secular trend in the most common male reproductive abnormalities including hypospadias, cryptorchidism, low sperm counts[1-5] and testicular germ cell cancer (TGCC)[6-10]. Over the past 30 years, the incidence of TGCC has increased substantially[10] and epidemiological studies predict that the incidence in Europe will increase by a further 24% by 2025[9 11].

The term 'testicular dysgenesis syndrome' (TDS) is often used to describe this group of individual disorders due to their frequent association and their relationship to impaired androgen production/action during fetal life. Genetic causes of impaired androgen action include androgen insensitivity syndrome (AIS), caused by a mutation in the androgen receptor in XY individuals, which results in under-masculinisation of the external genitalia, which can result in a typical female appearance in the complete (CAIS) form. AIS also includes features consistent with TDS (hypospadias, cryptorchidism, infertility and an increased risk of TGCC)[12 13]. Although it is clear that many TDS disorders can arise as a result of genetic abnormalities, there is increasing evidence that *in-utero* environmental factors (including lifestyle and chemical exposures) may also play a role (Figure 1)[3 14 15 16].

Studies in rodents have demonstrated that perturbation of androgen production/action during fetal life results in the development of TDS disorders in male offspring[17-22]. These studies involve *in-utero* exposure to a plasticising agent (Di-n-butyl phthalate; DBP) which results in subsequent cryptorchidism, hypospadias, impaired spermatogenesis and primary hypogonadism. Crucially, there is a critical window of sensitivity in fetal life, termed the 'masculinisation programming window' (MPW), during which the subsequent development of these disorders may be programmed by lack of androgen production or action[19]. The MPW has been shown to occur between embryonic day (e)15.5 – e18.5 in rats and has been estimated to correspond to 8-14 weeks of gestation in humans[19 22].

One of the challenges in understanding the development of these disorders in humans has been the assessment of fetal androgen exposure in male offspring. Measurement of anogenital distance (AGD), which is sexually dimorphic, can be used as a biomarker/read-out of fetal androgen exposure, and thus are a proxy for anti-androgenic effects of in-utero exposures[23-25] that can be utilised throughout life[25 26]. AGD is reduced in boys presenting during the neonatal period with cryptorchidism and/or hypospadias[27] and in adulthood, AGD has been shown to be positively associated with semen quality[28 29] and serum testosterone[23]. It should be emphasised that whilst measuring AGD can be used for carefully conducted population-based studies, technical difficulties, in terms of accuracy of measurement, reproducibility and time required to conduct this examination, limit its utility for clinical assessment of individuals.

The association between events that occur during fetal life and the development of male reproductive disorders, combined with the evidence to suggest a role for environmental influences has resulted in a large number of epidemiological and experimental studies investigating a range of chemical and pharmaceutical exposures. Recently a number of studies have described a potential role for analgesics in the development of these disorders[30-40]. In order to describe the potential effects of in-utero exposure to analgesics on male reproductive development we will first discuss in detail the development of the testis from fetal life focusing on cellular events that determine testosterone production and germ cell development. We will then briefly describe the approach to assessing in-utero environmental exposures before describing in detail the potential effects of paracetamol (the most commonly used analgesic worldwide) exposure during pregnancy, on male reproductive health in the offspring and subsequent generation(s).

Testicular Development and Function during Fetal and Early Postnatal Life

Formation of the testis

In order to understand the potential for *in-utero* exposures to affect the developing testis, it is important to understand testicular cellular development and function during fetal life and the early postnatal period (Figure 2). Primordial germ cells (PGCs) emerge at 4-5wks post conception in the human, and migrate from the yolk sac into the genital ridge where they become known as gonocytes[41]. Interaction between gonocytes and the stromal microenvironment, along with the presence of a single transcription factor coded by the Y chromosome, sex-determining region Y protein (SRY), determines gonadal sex. Following *SRY* expression, gonadal somatic cells, arising from the coelomic epithelium, begin to differentiate into Sertoli cells (SC)[42]. SC aggregate in a layer around gonocytes, thus forming the seminiferous cords at approximately 6-7 gestational weeks in the human (or e13 in the mouse)[43 44]. The seminiferous cords separate the seminiferous epithelium, consisting solely of germ cells and SC, from the interstitial compartment, which includes the testosterone-secreting fetal Leydig cell population[45].

Sertoli cell (SC) development and function

SC play a vital role in supporting spermatogenesis in the adult testis. The total number of SC in adulthood determines final testis size, number of germ cells and maximum sperm output[46 47 48]. However, SC also have important functions in fetal life including secretion of anti-Müllerian Hormone (AMH), necessary for regression of the Müllerian ducts, and factors (e.g. Desert Hedgehog) which induce fetal Leydig cell differentiation[49-52]. SC proliferation in humans occurs during fetal and neonatal life followed by a quiescent period until the second proliferative wave around puberty[48]. The switch from pre-puberty to puberty triggers the final maturation from proliferative immature SC to non-mitotic mature SC capable of supporting spermatogenesis (Figure 2). Rodent studies have indicated that prenatal exposures to plasticising chemicals known as phthalates can arrest SC development in their immature state, thus interfering with spermatogenesis[18 21] and foci of immature SC are also a common feature in infertile men with azoospermia and other TDS disorders[21 53].

Germ cell development and fertility

During fetal and early postnatal life, the germ cells differentiate from gonocytes into spermatogonia during which they cease to express pluripotency markers (e.g. OCT4, Nanog)

and begin to express germ cell-specific spermatogonial proteins (dDX4, MAGE-A4), a process that occurs asynchronously in humans from late second trimester to ~6 months postnatally[54]. This period of germ cell development is important in establishing the spermatogonial stem cell pool, not only for future fertility but also because failure of gonocytes to differentiate into spermatogonia during fetal/early postnatal life is thought to result in germ cell neoplasia in-situ (GCNIS), which is the pre-malignant step towards TGCC in adulthood[55] (Figure 2). In addition to the increased risk of TGCC, individuals with TDS disorders may also exhibit dysgenetic areas in the testis in which germ cells are lost and seminiferous tubules become Sertoli cell-only (SCO). Therefore, environmental influences that impair germ cell development during fetal and early postnatal life may result in reduced fertility and/or the development of TGCC[16].

Leydig cell development and function

Leydig cells (LC) are essential for endocrine function of the testis as they produce the steroid hormone testosterone. Testosterone is primarily required for fetal masculinisation (including testicular descent), pubertal development and spermatogenesis. LC development in the human is considered a 'triphasic event', as demonstrated by the peaks in plasma testosterone levels across the life course that coincide with the development of separate lineages of LC (Figure 2). A fetal LC population is present from 6 weeks gestation in humans (e12.5 in the mouse)[56-59]. By 2-3 months of postnatal life, a neonatal population is evident, responsible for the so-called 'mini-puberty'[60] and finally, an adult LC population arises at puberty and remains throughout adulthood[61]. Placental hCG initially stimulates testosterone production from the fetal LC and insufficiency in the former is associated with hypospadias and cryptorchidism[62], whereas during the neonatal period, LH is responsible for stimulating the peak in plasma testosterone levels[63 64]. In rodents, perturbations of fetal testosterone production (e.g. due to phthalate exposure) may result in some TDS disorders, including Leydig cell hyperplasia[62] and has also been shown to be associated with primary hypogonadism/compensated LC failure in adulthood[20 21]. Insulin-like factor 3 (Insl3) is another hormone produced by the Leydig cell which, like testosterone, plays a crucial role in testicular descent. Perturbations in Insl3 during the fetal period can also result in cryptorchidism in rodents[65 66].

Assessing the Impact of Environmental Exposures on Human Health

Studies aimed at investigating the impact of environmental exposures on human health largely consist of epidemiological studies in human populations and experimental animal studies. Whilst epidemiological evidence can demonstrate associations between exposures and occurrence of a condition, experimental studies can imply causation and demonstrate potential mechanisms. Critical assessment of the literature on effects of environmental exposures must take into account the strengths and limitations of these approaches (Table 1). We will describe the epidemiological and experimental evidence in relation to paracetamol exposure and the development of male reproductive disorders and in doing so we will illustrate some of the factors that need to be considered in order to critically assess such studies.

Effects of Exposure to Paracetamol on Male Reproductive Health

Maternal use of paracetamol during pregnancy

Avoidance of pain and pyrexia during pregnancy is important for fetal and maternal health and analgesics play an important role in the treatment of pain and pyrexia during pregnancy. In a large study of pregnant women from Europe, US and Australia (n=9,459), 81.2% reported taking over-the-counter (OTC) medicines[67]. It is reported that >50% of pregnant women in the US take OTC analgesics, including paracetamol[68 69]. Studies in the UK of self-reported medication use have shown that paracetamol is the most commonly used OTC medication during pregnancy, with 36% of pregnant women reporting paracetamol intake during the 1st trimester (n= 465/1305)[70] and 54% during the first 20 weeks of gestation[71]. In a similar Danish study, 47% of mothers reported taking paracetamol at some point during their pregnancy[30]. Paracetamol readily crosses the placental barrier with a mean half-life of approximately 3 hours[72 73] and its serum concentration is equivalent between mother and fetus following an oral therapeutic dose (1g)[74]. The number of epidemiological and experimental studies into the potential relationship between analgesic exposure and male reproductive disorders have been increasing over recent years[31].

Epidemiology of paracetamol exposure and male reproductive function

Epidemiological studies have reported associations between maternal use of analgesics and cryptorchidism in resulting male offspring[30 32 33 75]; however, this is not consistent across all studies[34]. Where associations exist, they appear to be restricted to exposure during the 2nd trimester or following prolonged exposure[35]. The timing of exposure in relation to the critical period for programming male reproductive development is likely to be important. One study has reported an association between cryptorchidism and paracetamol exposure during the human MPW (estimated to be 8-14 gestational weeks[19]), however importantly, this was only statistically significant for prolonged exposure of >4 weeks[32].

The reported association between exposure to paracetamol and cryptorchidism could be explained by a reduction in androgen (testosterone) production by the human fetal testis, and anogenital distance (AGD) has been shown to represent an 'indirect' postnatal readout of androgen exposure during fetal life[24]. A recent prospective birth cohort study showed that 40% of women reported using paracetamol during the first 28 weeks of pregnancy[36]. Exposure to a mixture of mild analgesics (including paracetamol and NSAIDS) was negatively associated with AGD in male infants (n=20; 3 months) which suggests reduced androgen signalling during fetal life. However, these results do not show a paracetamol-only induced effect and this may highlight the importance of exposure to 'mixtures' potentially resulting in additive effects which may be more representative of analgesic intake during pregnancy[36]. A similar study (n=434 male infants) reported an association between paracetamol exposure and reduced AGD from birth to 2 years which was independent of body size[37] and interestingly, this association was limited to paracetamol exposure during the proposed human MPW (i.e. 1st trimester). Importantly, all of the above epidemiological studies are based on self-reporting of analgesic use, determined in most cases by the use of retrospective questionnaires. The possibility of inaccurate recall or recall bias and lack of direct measurements of analgesic exposure are important limitations of these studies.

Experimental approach to assessing the effect of paracetamol exposure during fetal life on male reproductive function

A variety of experimental approaches have been utilised to determine the effect of paracetamol exposure on the fetal testis, particularly in relation to testosterone production. This includes *in-vivo* approaches in pregnant rodents[30 38 76 77] and *in-vitro* studies using fetal testis tissues from rodents[30 78] and humans[39]. Recently, a xenografting approach has been developed which can assess the effects of exposures on the human fetal testis in a more physiological manner than can be achieved *in vitro*[38].

To investigate potential paracetamol-induced effects during fetal life, pregnant rats were exposed to doses of 150, 250 or 350 mg/kg/day from e13-21. AGD was reduced in male offspring compared to untreated controls[30] with similar effects on AGD described in other studies[38 77]. However, a recent study using the same regimen as described above (350 mg/kg/day from e13-21) did not demonstrate a significant effect on AGD[76]. The timing of exposure in these studies coincides with the MPW; however, the doses (150-350mg/kg/d) used are higher than therapeutic doses (~60mg/kg/d) to which humans are usually exposed[38]. There are also likely to be large differences in the pharmacokinetics and metabolism of these agents in rodents compared to humans, making direct correlations from dose effects between species challenging.

In-vitro paracetamol exposure studies have also been described using fetal rat testis explants (e14.5) cultured for 3 days in media containing paracetamol (1µM)[30 78]. This resulted in a significant reduction in testosterone production, even at a concentration of 1µM, which is well below the therapeutic concentration described in human plasma (65-130µM) following paracetamol ingestion[30]. This could suggest that *in-vitro* effects may under-estimate the effects that occur in humans, however it should be emphasised that circulating paracetamol levels may not be a direct indicator of intra-testicular levels. In addition, the in-vitro models cannot mimic the pharmacokinetics, including peak and trough concentrations that occur *in-vivo*.

The majority of experimental studies in rodents suggest a negative association between paracetamol exposure and fetal androgen production. This has prompted subsequent *in-vitro* studies using human fetal testis tissue. Exposure of first trimester testis (8-12 weeks gestation) to paracetamol (10 μM) for 1 to 3 days did not alter testosterone production compared to vehicle-exposed controls[39]. The differences between the results of the rodent and human *in-vitro* studies may relate to differences in the suitability of the *in-vitro* system or the timing of exposure in relation to the stage of development (including the timing of the MPW) between these two species[39]. *In-vitro* culture conditions cannot replicate normal human testosterone production or the *in-vivo* environment. To circumvent some of these limitations, an *ex-vivo* xenograft approach can be undertaken to investigate potential paracetamol-induced effects on the human fetal testis[79 80]. In this system, human fetal testis tissue pieces (n=5; 14-20weeks gestation) are grafted subcutaneously under the dorsal skin of castrate host nude mice. Paracetamol was then administered orally to the host animal according to a therapeutic regimen (20mg/kg; 3 times daily), resulting in a significant reduction (45%) in host serum testosterone after 7 days' paracetamol exposure, whilst a single day's exposure did not affect testosterone production[38]. Interestingly, plasma paracetamol concentrations, measured 1 hour after the final dose[38] were significantly lower compared to post-therapeutic levels reported in pregnant women[72] suggesting that the effects on testosterone can occur at clinically relevant paracetamol doses/exposures. The effect of prolonged paracetamol exposure of xenografts[38] is in contrast to the results obtained from *in-vitro* culture of human fetal testis tissue[39]. This could be due to the method of delivery (media versus oral administration), timing of exposure *in-vitro* (8-12 weeks gestation) compared to xenografts (14-20 weeks gestation), the relative dosage, length of exposure or difference in metabolism between *in-vivo* and *in-vitro* studies. Further limitations of both the *in-vitro* and xenograft systems include the lack of a feto-placental unit, although it has been demonstrated that paracetamol is able to cross the placenta and enter the fetal circulation in similar concentrations to maternal plasma[72 73].

Overall, the results of the experimental studies suggest that exposure to paracetamol during fetal life may lead to a reduction in testosterone production in humans, although direct correlation between the degree and duration of reduced testosterone and the potential to develop male reproductive disorders cannot be made from these studies. Whilst the majority of circulating paracetamol in humans comes from ingestion of paracetamol

containing medications, an alternative source of paracetamol has also recently been described. The industrial chemical aniline, which is found in a wide variety of manufactured products e.g. pharmaceuticals, cosmetics, cigarette smoke, has been shown to be rapidly metabolised to paracetamol inside the body[81 82]. *In-vivo* studies involving *in-utero* exposure of male mice to aniline have shown similar fetal anti-androgenic effects to those described for exposure to paracetamol[77].

It is known that analgesics, including paracetamol, can inhibit prostaglandin synthesis[83]. During fetal life, germ cells have been shown to be prostaglandin targets[84 85] indicating that germ cells may also be susceptible to analgesic-induced changes, which in turn may alter GC development and fertility in the offspring. To investigate this, *in-vivo* studies of *in-utero* paracetamol exposure have been conducted in rodents[85 86]. In one study, pregnant rats were exposed to paracetamol (350mg/kg/d) from e15.5-18.5, which resulted in altered germ cell development in both sexes of offspring[85]. In males there was an accelerated loss of expression of a pluripotency marker (OCT4), suggesting germ cell differentiation from gonocyte to spermatogonia had occurred prematurely. It is interesting to note that the effects on germ cell differentiation in F1 males occur during the MPW, a critical period for male reproductive development[21 85]. In F1 females there was a delay in meiotic entry in oogonia. After *in-utero* paracetamol exposure, female offspring had reduced ovary size and exhibited reduced litter size in adulthood, whilst no reproductive effects were seen in males, suggesting a potential compensatory mechanism between fetal life and adulthood[85]. In another study, exposure of pregnant rats to a much lower dose of paracetamol (50mg/kg/d) from 7 days post-conception to birth resulted in a reduction in follicle number at 2 months postnatally and also a reduction in pups per litter at 6 months of age[86].

During fetal life, germ cells undergo epigenetic reprogramming[87] which suggests that any perturbations to germ cells during this period could have inter-generational effects. The potential for such effects occurring following *in-utero* paracetamol exposure was investigated in a recent study in which paracetamol-exposed (male and female) mice were mated with a non-exposed partner. The subsequent F2 females had reduced ovary weight

(as previously described) and reduced number of primordial follicles, whilst F2 males did not appear to have any demonstrable germ cell effects[85].

Postnatal effects of paracetamol exposure on male reproductive function

Whilst the majority of studies have investigated the effect of analgesic exposure on the fetal testis, one study has demonstrated that the adult testis may be susceptible to endocrine perturbations following paracetamol exposure. Explants from human adult testis (average age: 79.8 ± 5.6 years) were cultured *in vitro* with therapeutically relevant doses of paracetamol for 24 hours, resulting in a significant reduction in testosterone production in the presence of 10^{-4} or 10^{-5}M paracetamol (30% and 18% reduction respectively)[88]. To date no studies have been conducted to assess the effect of analgesic use in adult men in terms of testosterone production or testicular function.

Analgesics are also commonly used in paediatrics; however, to our knowledge, there have been no studies investigating the effect of analgesic exposure during the neonatal, infancy or childhood period in terms of later male reproductive function. In addition, the testicular effects of exposures on babies born pre-term has not been investigated. Pharmacokinetic studies have shown that based on body weight, neonates have approximately 30% paracetamol clearance capacity compared to adults[89-91]. Exposure during the neonatal period may be of particular importance given the programmed rise in testosterone during mini-puberty[64]. Whether paracetamol exposure during this period can suppress testosterone and what the implications might be for subsequent reproductive health is unknown, although it is worth pointing out that in individuals with conditions such as hypogonadotrophic hypogonadism (in whom there is no rise in testosterone during mini puberty or at expected time of puberty), testicular function and fertility can be restored with exogenous gonadotrophins[92]. Given the relative quiescence of the HPG axis during the childhood period, it would be anticipated that the potential for anti-androgenic effects of analgesics would not be as important during this period compared to other stages throughout life; however, it is unknown whether exposures can affect Leydig, Sertoli or germ cell development during this period. Further studies are warranted to investigate the effects of exposure to analgesics during postnatal life.

Conclusion

Male reproductive disorders are common and some are increasing in incidence. These disorders can be associated and linked to a reduction in testosterone during fetal life. *In-utero* exposure to environmental agents are likely to play a role in the development of these disorders. In order to assess the potential impact of environmental agents on male reproductive function in humans, careful consideration of study methodology is required, particularly in relation to the model system and species, in addition to dose, route and duration of exposure. Effects of exposures, e.g. reduction in testosterone, must be considered in the context of the potential to result in a subsequent disorder such as cryptorchidism or reduced sperm production/counts. For *in–utero* paracetamol exposure, epidemiological studies demonstrate associations with cryptorchidism in the offspring, primarily in relation to exposures during the second trimester and prolonged duration of exposure, whilst experimental studies suggest that paracetamol can reduce testosterone production by the human fetal testis. Based on the current evidence discussed, it cannot be concluded that exposure to paracetamol is a direct cause of male reproductive disorders nor that analgesics should simply be avoided during pregnancy. The importance of managing pain and pyrexia during pregnancy should also be considered for the health of the mother and fetus. As a result, a pragmatic approach is to ensure that where analgesics are deemed to be necessary, that they are used at the minimum therapeutic dose for the shortest possible duration. Future studies should aim to determine the clinical relevance of the findings related to *in-utero* exposures, in addition to investigating the effects of exposures during postnatal life.

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Conflicts of interest

None

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Figure Legends

Figure 1 – In-utero factors proposed to predispose to Testicular Dysgenesis Syndrome (TDS) disorders. Many lifestyle, genetic and environmental factors during fetal life have been postulated to lead to the development of TDS disorders in male offspring, albeit with variable (and in some cases limited) sources of evidence[16].

Figure 2 – Testicular development in health and disease. Development of germ (green panel), Leydig (yellow panel) and Sertoli (purple panel) cells during different stages of testicular development. Differentiation of germ and Sertoli cells (dotted line) and the individual Leydig cell populations are shown. Note that for germ cells, failure of differentiation from gonocyte to spermatogonia leads to the development of pre-malignant Germ Cell Neoplasia In-Situ (GCNIS) cells which results in testicular germ cell cancer in young adulthood. The Masculinisation Programming Window (MPW; shaded box) is shown during fetal life in which reduced testosterone may result in the development of Testicular Dysgenesis Syndrome (TDS) disorders. Relative testosterone production during developmental periods is also indicated (black line).

Tables

Epidemiological
Study Design - descriptive, case-control or experimental, relevant population
Exposure - measured directly (agent) or indirectly (questionnaire, proxy exposure)
Effect - direct effect (e.g. cryptorchidism) or indirect (AGD)
Other - mechanistic plausibility for effects

Experimental
Model - species, experimental system (e.g. *in-vivo, in-vitro, ex-vivo*)
Agent - same agent or similar class/compound, metabolism of agent
Dosing - dose equivalent (e.g. serum/*in-vitro* concentrations), timing, duration
Effect - direct effect (e.g. cryptorchidism) or proxy for clinical condition (AGD)
Mechanism - e.g. effect on signalling pathways (including rescue)

Table 1 – Key points to consider when assessing epidemiological and experimental studies relating to the effects of environmental exposures on male reproductive development.

Response to Reviewers

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
The paper is written by a research group which has been very active in research on impact of paracetamol on male development. It is often useful when researchers reflect on their recent results in an analysis of available data and put them into perspective. While I like the discussions on paracetamol I feel that the general aspects of the topic, as the title seems to suggest, are somewhat superficially analysed. I suggest that the title 'Assessing the impact of in-utero environmental exposures on male reproductive function and fertility' is changed to reflect this limitation of the paper and focus more on paracetamol.

We thank the reviewer for their helpful comments and we have taken these on board in the revised manuscript. It is important to bear in mind that this review was written with the ADC readership in mind and therefore is aimed at a general paediatric audience with the intention of introducing the concepts of assessing endocrine disruption using exposure to analgesics and impacts on male reproductive function to illustrate these points. We have however altered the text to make it clear in the introduction that the focus is on paracetamol (P1, Line 24; P3, Line 79-82). We have also altered the title to reflect this.

Specific points:

1) As the authors relate their findings to human TDS, the description of TDS seems to lack some important reported findings in these men, including the persistent dysgenetic, histological changes in the Sertolic and Leydig cells, which can be seen in adult men with the disorder (as described by Rajpert-De Meyts E. Hum Reprod Update. 2006).

This is an important point and we have included this in these points in the manuscript (P 5, Line 126-128).

2) The reader may get a wrong impresion of the doses of paracetamol generally used. On page 7, line 55 they write:' ..... however, the doses (150-350mg/kg/d) used are higher than human therapeutic doses (~60mg/kg/d) to which humans are usually exposed.' This stqatement is not correct. 60mg/kg/d is a maximal 24 h dose, only used in severe pain. Unfortunately, previous papers have also exaggerated the doses of paracetamol most often used.

We respectfully disagree with this point. Paracetamol doses in children in the UK are prescribed according the British National Formulary. The therapeutic dose for pain/pyrexia is an initial loading dose of 20mg/kg and 10-15mg/kg 4-6hrly (maximum 4 doses/24 hrs). This equates to 60mg/kg/d and does not specifically apply to 'severe' pain. There may be variations in paracetamol dosing in different countries to which the reviewer is referring but it is reasonable to describe the stated doses as 'therapeutic dosing'. In addition, in xenograft studies paracetamol levels have been directly measured in the serum of the host mouse and have been shown to be below those measured in the serum of humans following a therapeutic dose.

3) Although the reader may expect from the introduction that farmaceutical paracetamol exposure will be discussed in relation to other exposures, mentioning of other exposures

e.g. exposure via secondary (non-pharmaceutical) sources, such as metabolic conversion from the ubiquitous industrial compound aniline are not well presented (although refences are mentioned). Neither the possibility that paracetamol effects may depend on simultaneous exposures from environmental chemicals, e.g. phthalates, bisphenol A, and UV filters.

As discussed this review was intended to use paracetamol as one example of how the effects of exposure to endocrine disruptors are assessed. We have altered the manuscript (and the title) to reflect the fact that we do not discuss other 'EDCs' in detail. We have also mentioned the concept of 'mixtures' in relation to analgesic combinations (Page 7, Line 200-202). We agree that reference to other sources of paracetamol (e.g. aniline) is important and we have therefore included text (Page 10, Line 274-281) to highlight this.

4) Table 1 contains numerous important keywords of which only few are discussed. Furthermore it lacks a third group of studies where humans participates in endocrine studies with examinations of body fluids and cells (such studies are not just 'epidemiological'). Either the table legend should be expanded or the table deleted and replaced with a couple of sentences.

We agree that 'clinical studies' involving human participants are important approaches to the study of environmental and pharmaceutical exposures. However, they can still be broadly classified either as an 'experimental' or 'epidemiological' study depending on whether an intervention (experimental) was involved. Descriptive studies relating to exposures (including measured levels in bodily fluids) and associations with clinical outcomes etc can be classified as epidemiological studies. We have made some adjustments to the legend and discussed these factors throughout the text.

5) Table 2 is also difficualt for an outsider to understand. It needs much more explanation (meaning of crosses?) and two symbols in a box is difficult to understand. The text should be expanded.

We agree that Table 2 will be difficult to understand so we have deleted the table. The points raised in the table are discussed throughout the text.

6) Fig 1. is somewhat misleading. If the authors believe that over/undernutrition can cause TDS they should include data. Anti-androgenic and estrogenic chemicals are mentioned. However, this whole field of endocrine dsirupters has expanded during the past 10 years. The figure text could perhaps be used in a high school text book, but not in a scientific journal as Archives of Diseases in Childhood.

We agree that the list of 'potential' endocrine disruptors has increased dramatically over recent years and that this should be reflected in the text of the figure and we have modified this accordingly. The purpose of this introductory figure is simply to get across to a primarily general paediatric audience the idea that many exposures from a variety of different sources have been implicated as playing a role in the development of TDS disorders. We use ?? to highlight the fact that for many of these exposures their role in the development of TDS is still not clear.

7) Fig. 2 is somewhat confusing. The link between the gonocyte and germ cell cancer is not easy to spot. The group has previously produced TDS figures which were more helpful.

This figure is intended to describe testicular development across the lifecourse rather than to focus completely on mechanisms relating to TDS, hence the differences compared to our previously published figures on TDS origins and mechanisms. We refer to publications relating to TDS in which such figures are available. We agree with the reviewer that the relationship between gonocyte and GCNIS/TGCC is not illustrated in the current figure and we have modified the figure to explain this concept.

Reviewer: 2

Comments to the Author
This is a well written review and I have very few comments because of the quality of the writing and content

We thank the reviewer for the positive comments.

Page 3, line 14 – do the authors mean technical or practical difficulties? – presumably they simply mean that measuring this distance is embarrassing and not acceptable to humans? Is that correct?

The measurement of AGD is technically difficult in terms of accuracy of measurement and reproducibility as it depends on the expertise of the examiner and also requires accurate calibrated equipment. Practical difficulties relate to time required to measure and acceptability to patients in addition to clinical applicability for individual patients. We have modified this sentence to account for this (Page 3, Line 66-69).

Page 8, line 15 – presumably one form of exposure (in vitro) is continuous whereas in human plasma there are peaks and troughs?

This is correct and we have made reference to this in the text (Page 8, Line 238-242).

I think that using paracetamol as an illustration of the issues involved generally works. However the discussions are primarily about paracetamol and testosterone – which is (it can be argued) not of greatest interest because many of its' key effects occur following conversion to dihydrotestosterone or oestrogen. Could the authors discuss or comment?

It is true that DHT mediates some of the key masculinising effects in the male (e.g. external genitalia); however it is clear from rodent studies and clinical conditions that reductions in testosterone can result in TDS-like and masculinisation disorders which reflects the fact that testosterone is the primary precursor for DHT and oestrogen. Previous *in-vivo* rodent studies, investigating the effect of blocking both testosterone and DHT (via flutamide) in male fetuses results in subnormal AGD which correlates with incidence and severity of hypospadias, cryptorchidism, and reduced phallus length and seminal vesicle weight in males (Welsh et al 2008). We initially refer to androgens which would include DHT and testosterone, before focusing on testosterone which is known to be important for testicular descent.

I did wonder if readers would find a table listing the key agents that have been implicated in the evolution of disorders of reproduction / fertility of value .

We have increased the list in Figure 1 of EDCs that have been proposed to cause TDS disorders in response to this comment and that of Reviewer 1.

Page 11 line 10 – some are increasing…(remove ‘at least’)

This has been removed as requested.

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Mechanism - e.g. effect on signalling pathways (including rescue)

Table 1 – Key points to consider when assessing epidemiological and experimental studies relating to the effects of environmental exposures on male reproductive development.

??

Lifestyle Factors

- Maternal smoking/ alcohol consumption
- Sedentary work/lifestyle
- Over/under nutrition
- Stress

Genetics

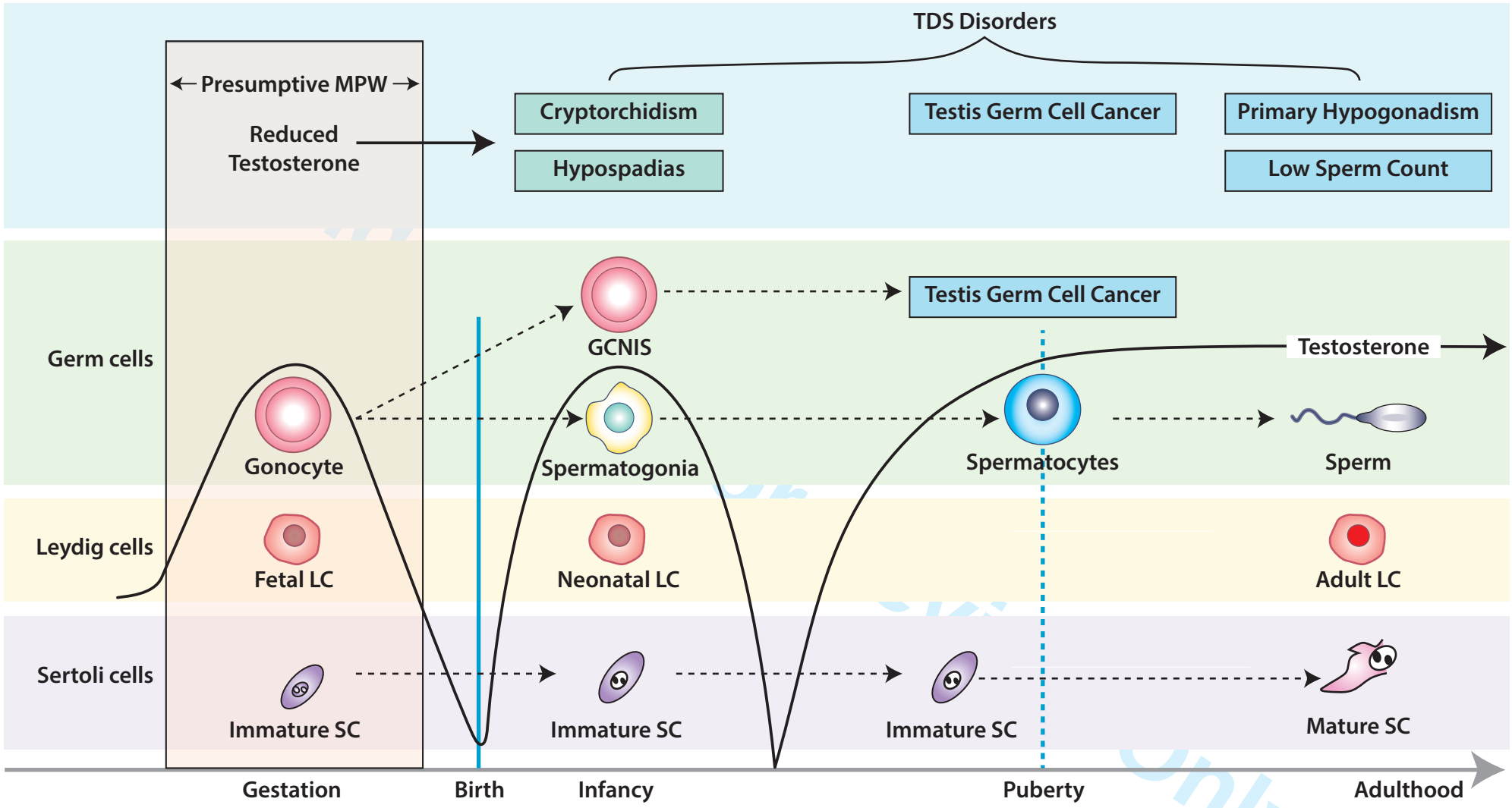
- Genetic Mutations
- Epigenetic Factors
- Fetal Growth

Environmental exposures

- Pharmaceuticals e.g. analgesics
- Endocrine disrupting chemicals/anti-androgenic or estrogenic:
- Pesticides
- Phthalates
- Dioxins
- Perfluorinated compounds
- Ultraviolet filters

TDS Disorders

- Cryptorchidism
- Hypospadias
- Testicular Germ Cell Cancer
- Low Sperm Count
- Primary Hypogonadism


TDS Disorders
Presumptive MPW
Reduced Testosterone
Cryptorchidism
Hypospadias
Testis Germ Cell Cancer
Primary Hypogonadism
Low Sperm Count
Germ cells
Leydig cells
Sertoli cells
Gonocyte
GCNIS
Spermatogonia
Spermatocytes
Sperm
Testosterone
Fetal LC
Neonatal LC
Adult LC
Immature SC
Immature SC
Immature SC
Mature SC
Gestation
Birth
Infancy
Puberty
Adulthood